Role of Heme oxygenase-1 in Murine Renal Allograft Acceptance

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by

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Abstract:

The acceptance of renal grafts in mice seems to be strain specific. While a kidney transplant between MHC disparate Balb/C and C57/B6 mice is rapidly rejected, a DBA kidney transplanted into a B6 mouse is spontaneously accepted and the acceptance is long term. Examination of sera from the B6 recipient demonstrates graft reactive antibodies suggesting an immune response being mounted. Yet the graft remains functioning suggesting that a state of tolerance has developed.

Many theories as to the mode of immune regulation have been proposed including the presence of regulatory cytokines (IL-10 and TGFbeta), and the presence of regulatory T-cells. Regulatory T-cells (Tregs) can be identified by the expression of the forkhead transcription factor foxP3, but the mechanism by which these Tregs down regulate the immune response remains to be determined. One theory is that foxP3 up-regulates the expression of the enzyme heme oxygenase-1 and this molecule produces physiological levels of carbon monoxide which has been shown to down regulate the activity of graft reactive T-cells. Therefore, we hypothesize that the regulation of the accepted grafts act through the presence of FoxP3 positive regulatory T-cells acting through up-regulation of heme oxygenase-1.

To test this hypothesis we isolated RNA from accepted DBA to B6 renal allografts at various time points (day 0, 30, 60, 150). After reverse transcribing the RNA and using a polymerase chain reaction to clone heme oxygenase-1, expression of the gene was compared to expression of heme oxygenase-1 in B6 to B6 murine renal isografts taken at the same time points using gel electrophoresis. The expression of FoxP3 was

also measured using the same procedure as stated above. This study is important because the DBA to B6 model seems to mimic the human condition, as patients have been seen that express enough antibody to warrant rejection but are not rejecting their renal grafts after transplantation.

Introduction:

Major histocompatibility complex (MHC) class I molecules are glycoproteins expressed on the cell surface of every nucleated cell. Structurally speaking, MHC class I molecules consist of an alpha chain or heavy chain (44-kDa) noncovalently associated β2-microglobulin or light chain (12-kDa) [1]. They are coded by the A, B, and C loci in humans and the K and D regions in mice located on chromosome 6 in humans and on chromosome 17 in mice[2]. In humans the proteins coded by these loci are called HLA proteins and several[2] polymorphisms have been found: 263 HLA-A, 501 HLA-B, 125 HLA-C, 6 HLA-E, 1 HLA-F, and 15 HLA-G[1].

By binding different endogenous peptides digested in the cytosol and presenting them to $CD8^{+}$T cells [1], 1MHC molecules act in intercellular recognition and discrimination between self and non-self cells. In doing so, they develop the humoral and cell mediated immune responses. The MHC haplotype determines what serum antibodies are made by the organism. When they are made in the thymus, T cells with receptors resembling these self-antigens are removed during processing. Therefore there the T cells present in the cell will only bind foreign antigens and recognize each self antigen as belonging to the organism. T cells bind to antigenic peptides which are bound to the 1MHC molecules[2]. Killer inhibitory receptors, KIRs, also bind to peptides held by these

molecules. Therefore the role of 1MHC is to facilitate the interaction of antigens with T cells or KIRs[1].

Inbred strains of mice are homozygous at all of the MHC loci. Therefore they are histocompatible and graft transplants can be done between them without eliciting an immunological response from T cells. When two strains having different MHC haplotypes are transplanted they induce an immune response, meaning they are histoincompatible and graft rejection occurs[2]. The foreign MHCs expressed on the graft are recognized by sera containing anti-"graft" antibodies. These antibodies bind to the graft endothelium and smooth muscle, which cause a signal to be sent trough out the cell, causing cell activation and proliferation[1]. These cell changes cause acute and chronic rejection.

Rejection of a graft occurs in two stages: the sensitization stage and the effector stage[2]. During the sensitization stage antigen presenting cells (dendritic cells, endothelial cells) of the host endocytose damaged proteins from the graft, including MHC molecules, and presents these proteins as "foreign" to T-helper (T_H) cells of the recipient. These cells in turn activate the immune system to mount an immune response toward the "foreign" graft[2]. The effector stage begins once T_H cells secrete cytokines (IL-2, IFN-γ, TNF-β) which generate effector mechanisms causing an influx of T cells (both T-helper cells and cytotoxic T cells) and macrophages into the graft. These effector mechanisms include a cellular (cell mediated) response and a humoral (antibody mediated) response (including antibody/complement lysis, and antibody-dependent-cell-mediated cytotoxicity). The cellular response is defined by the influx of macrophages and cytotoxic T-cells which invade and attack the graft. T-helper cells aid in this by

expressing Il-2, causing T cell proliferation and generation of cytotoxic T-cells, IFN-γ caused inflammation and activation of invading lymphocytes, and TNF-β guides the cytotoxic effect. Many cytokines including IFN-α, IFN- β, IFN-γ, TNF-α, and TNF-β increase the expression of MHC molecules allowing for greater recognition by T cells which in turn amplifies the response.

The humoral response is defined by antibody binding to graft cells and activating the complement cascade or signaling destruction of the cells by neutrophils and macrophages. Antibody activation of the complement cascade causes a series of chemical reactions on the surface of a cell resulting in cell lysis and death. Further, complement activation causes the infiltration of macrophages and lymphocytes which in turn amplifies the cellular arm of rejection. Signs of chronic rejection are accelerated transplant arteriosclerosis, ischemic injury, and decline of organ function[1].

In some instances, however, chronic rejection is not seen despite the presence of antibody and other inflammatory mediators. Such survival in the face of a pro-inflammatory/rejection state is termed “accommodation”. In several case reports, patients have graft reactive antibodies and a pro-inflammatory graft yet do not reject the organ[3]. The rationale for this is uncertain but may be due to signaling of protective mechanisms in the graft by antibody. It has also been shown that antibodies binding to MHC molecules on cells signal pro-inflammatory and proliferative phenotypes. Antibody-MHC binding induces the expression of FGFR in endothelial, smooth muscle, and epithelial cells. The cell is now responsive to FGFs triggering the activation of the ERK/MAP kinase pathway, cyclin E-dependent kinase activity, and Rb inactivation, which all cause cell proliferation[1]. In several studies, endothelial cell (EC) exposed to

sub-saturating concentrations of MHC class 1 antibodies were resistant to activation and Ab/complement-mediated cell death[3]. The protective mechanism seemed to be mediated through the up-regulation of anti-apoptotic genes including Bcl-xL, Bcl-2, and heme oxygenase-1.

It is believed that heme oxygenase-1 (HMOX-1) is one enzyme through which tolerance is achieved. Heme oxygenase-1 is the enzyme which catalyzes the degradation of heme releasing iron, carbon monoxide (CO), and biliverdin[5]. It has been found that transfection of HMOX-1 into Jurkat T cells inhibits cellular proliferation and is thought that this phenotype is mediated by the production of CO. CO has been shown to exert antiproliferative effects on vascular smooth muscle cells and T cells by blocking the production of IL-2[5]. CO also inhibited caspase- 3 and caspase-8 activity in T lymphocytes by the up-regulation of $p21^{Cip1}$, a cell cycle inhibitor[6].

Heme oxygenase-1 is believed to exert its regulatory effects through a specific type of T-cell, the regulatory T cell (or the Treg). Regulatory T cells or Tregs have been shown to suppress immune responses by inhibiting the reaction of autoreactive T cells in a cell contact dependant manner. Tregs specifically express FoxP3, which encode scurfin (also known as forkhead box P3), a transcription factor. FoxP3 expression has been shown to convert naïve $CD4^{+}CD25^{-}$ T cells into Tregs (CD4+CD25- T cells) and is therefore imperative in their development[4]. Thus T-regs may mediate accommodation through the expression of heme oxygenase-1.

We sought to study this phenomenon in a murine renal transplant model. In our studies, we have found that a kidney transplant between MHC disparate Balb/C and B57/B6 mice is rapidly rejected as expected. However, a DBA kidney transplanted into a

B6 mouse is spontaneously accepted even though HLA antibodies are found in the B6 sera. We hypothesized that FoxP3+ Regulatory T cells (Tregs) in the B6 mice with a grafted DBA kidney could be suppressing an immune response through the up-regulation of heme oxygenase-1 and allowing for accommodation despite high anti-graft antibody levels.

Methods:

Transplant:

A DBA murine kidney was transplanted into a C57/B6 mouse. Because the left kidney has a longer renal vein than the right kidney, it is removed from the donor to use as the graft tissue. Prior to receiving the graft kidney, the native right kidney was removed from the recipient. An end to side anastomosis (attachment of 2 vessels) between the donor renal vein and the recipient inferior vena cave was performed. Then the donor aortic cuff was attached to the recipient aorta via an arterial anastomosis. The urinary tract was reconstructed using a bladder patch to suture the donor ureter to the recipient bladder. Each mouse was anesthetized with 65mg/kg of pentobarbital during surgery and was administered 0.04mg/kg of Atropin by subcutaneous injection before anesthetization. The graft was washed several times with saline through out reconstruction. A heat lamp was used during surgery and twenty-four hours post surgery to prevent hypovolemic shock. After surgery mice were treated with 500u/10g of penicillin in order to prevent infection from leaks in the bladder. Seven days post transplant the left kidney was removed, leaving the recipient with only one working kidney, the transplanted graft. Therefore graft function could be measured. Removal occurred after seven days in order

to allow the graft time to recover from ischemic injuries that occur during transplantation, reduce the trauma and blood loss of the recipient, and to give the micro-surgeon a chance to observe the graft[11]. Grafts were then collected on day 30, 60, and 150.

RNA Isolation:

The TrIzol Reagent Method (Ivitrogen) was used to isolate RNA from the renal samples. Tissues were homogenized in Trizol reagent (1ml) using a homogenizer. In order to complete dissociation of nucleoprotein complexes, the samples were allowed to sit for 10 minutes at room temperature. The Trizol reagent dissolves cells while maintaining the structure of the RNA. Chloroform (0.2ml) was added and the tubes were centrifuged to separate the homogenized solution into two phases: an aqueous phase containing the RNA and an organic phase. After transfer of the aqueous phase the RNA was precipitated using isopropyl alcohol (500ul). Glycogen (5ul) was added as a carrier molecule, aiding in precipitation (out competes RNA and saturates the solution so more RNA is precipitated). Once centrifuged, a gel like pellet forms. The pellet was air dried for 5-10min and resuspended in TE. Using a microspectometer the RNA was quantitated at 260 and 280. Only RNA with an A260/A280 ratio greater than 1.7 was accepted as clean and isolated.

Reverse Transcription

RNA was converted to cDNA using the Omniscript RT kit from Qiagen according to manufacturer's protocol. 2ug of each sample was added to a mastermix consisting of water, buffer, dNTP, Oligo-dT_{12-18} primer, RNase Out, and Omniscript reverse

transcriptase. The reverse transcriptase reaction commenced at 37°C for 60 minutes and samples were stored at -20°C until gene specific PCR reactions were performed.

PCR:

Semi-quantitative end point PCR (SuperArray) was performed by using a Programmable Thermal Controler-100 (MJ Research Inc.) to run a super array, consisting of 1 cycle of 15 minutes at 95°C to activate the HotStart DNA polymerase, followed by 30 cycles of 15 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C. Propriatary PCR primers for Hmox-1 or foxP3 and GAPD were used per company instructions.

Gel Electrophoresis:

PCR products were separated on a 2% agarose gel and visualized using ethidium bromide on a UV transilluminator. Gel images were captured using a Kodak DC120 digital camera, and analyzed using Kodak 1D-gel analysis system 3.6.0. Band intensity was determined for each sample and normalized to a GAPD housekeeping gene band run with each sample. Data is reported as normalized intensity for a given sample.

Statistical analysis:

Data is reported as mean ±S.E.M. Statistical significance was determined by a non-parametric ANOVA followed by a Dunn's post-hoc test for individual differences. Correlation analysis was performed using a Spearmann Rank correlation coefficient. Significance was set at $p<0.05$.

Results:

We examined HMOX-1 and FoxP3 expression at 4 different time points for murine DBA to B6 renal iso- and allo grafts. A typical gel is shown in figure 1. As seen in figure 1a. HMOX-1 runs at 143 base pairs and GAPD is seen above it at 435 base pairs. As seen in figure 1b. HMOX-1 runs under GAPD at 132 base pairs. There seems to be no difference between samples.

Expression of heme oxygenase-1:

In comparing expression of heme oxygenase-1 the following results were obtained as seen in figure 2. At the time of transplantation there was no statistical difference between expression of HMOX-1 in B6 and DBA mice. For normal B6mice (d0, n=3) the mean expression of HMOX-1 was 0.9872 (+/-.2197) relative units, while normal DBA mice (d0, n=3) showed a mean expression of 0.6833 (+/- 0.3409) relative units. All measurements are expressed in relative units to GAPD. Isografts (n=3) at day 30 showed a mean expression of 0.8524 (+/-0.2245) relative units and allografts (n=3) of day 30 showed a mean expression of 0.9872 (+/-0.2197) relative units. Day 60 isografts (n=3) showed 0.8684 (+/-0.2505) relative units of expression versus 0.9189 (+/-0.7847) relative units for allografts (n=4). By day 150 expression was 0.3994 (+/-0.3048) and 1.037 (+/-0.5850) relative units for iso (n=3) and allo (n=4) grafts respectively. Therefore no statistical difference in the expression of heme oxygenase-1 was seen at any time point between the iso- and allo-grafts. Spearmann Rank correlation coefficient test was run and no correlation was found either.

Expression of foxP3:

After calculating the mean of expression in relative units for FoxP3 no statistical difference was found between allo- and iso- grafts of DBA to B6 mice as seen in figure 3. The means of expression for the different time points are as follows: d0: DBA$_{(n=4)}$ = 0.2568 (+/- 0.3426), B6$_{(n=3)}$ = 0.2303 (+/- 0.1248); d30: allo$_{(n=3)}$ = 0.1454 (+/- 0.2518), iso$_{(n=3)}$ = 0.5009 (+/- 0.5887); d60: allo$_{(n=4)}$ = 0.1408 (+/- 0.2816), iso$_{(n=4)}$ = 1.416 (+/- 1.136); d150: allo $_{(n=5)}$ = 0.6717 (+/- 0.9173), iso $_{(n=3)}$ = 0.0 (+/- 0.0). All time points were measured in relative units to GAPD. No correlation between time points was found after analysis by a Spearmann Rank correlation coefficient test.

Discussion:

Graft accommodation in Allo-grafts of DBA to B6 kidney mice was thought to occur because of down regulated activity of T cells from low levels of carbon monoxide produced by HMOX-1 up-regulated by FoxP3. The data above does not support this theory as there is no significant difference between the expression of HMOX-1 or FoxP3 in the iso- and allo-grafts. This does not mean that a difference does not exist nor that accommodation does not occur via this pathway. We looked at the entire renal tissue so the concentration may be too small to show small differences between HMOX-1 and FoxP3 expression in the cell types. Perhaps a difference between expressions may have been seen if we had the ability at the time to isolate graft infiltrating cells.

Four different time points (d0, d30, d60, d150) were looked at for graft expression of HMOX-1 and FoxP3. Perhaps the role of heme oxygenase-1 and thus foxP3 occurs

directly after transplantation before the day 30 time point. They may be important in protection against early antibody-mediated injury and were not assessed in this study. Our results were similar to the ABO-incompatible kidney allograft accommodation study by Park et. Al. in which they demonstrated that heme oxygenase-1 was not increased in accommodated ABO-incompatible grafts at 1 year and 3 months after transplantation[9]. Likewise they did not assess the grafts early after transplantation, so perhaps HMOX-1 protects the graft during an early time period.

Fantini et. al. demonstrated that FoxP3 was induced in peripheral murine T cells causing them to become $CD4^{+}CD25^{+}$ T regulatory cells, so immunological tolerance was obtained[10]. We did not see an up-regulation of FoxP3. Fantini studied T cells using cell culture of splenic T cells from FVB mice. The environment of cells in culture is much different than their environment in a tissue. Perhaps these differences cause a change in the way cultured cells react to immunological stimuli and so they do not react as a typical tissue cell would. Spleen cells may also react differently than kidney cells for some unknown reason.

It is possible that accommodation acts through some other molecule. Many studies have indicated that indoleamine2,3-dioxygenase (IDO) may play a role in immunological tolerance as well. Expressed in monocytes, macrophages, and DCs, IDO is the enzyme that converts tryptophan to kynurenine, producing the byproducts 3-hydroxyanthranilic acid (HA), picolinic acid, and quinolinic acid[5]. Two mechanisms of immunoregulatory responses have been proposed. First, studies have shown that a depletion of tryptophan, an essential amino acid, may reduce T cell proliferation. The second suggests that the byproducts of the IDO-tryptophan reaction are proapoptotic or

antiproliferative to T cells[5]. HA, a byproduct of IDO was shown to up-regulate HO-1 expression by Oh. et al. The CO produced from this up-regulation inhibited inducible NO synthase (iNOS) expression and NO production causing a cytoprotective effect. A positive feedback loop was seen as CO was found to up-regulate IDO. However this experiment was not done on Treg cells but RAW264.7 macrophages stimulated with LPS and IFN-γ, so this theory would need to be verified in Tregs. Jurkat T cells have also been shown to up-regulate HO-1 expression when NO donors were added to the medium, supporting the above theory. It has also been found that activated T cells give off signals such as CTLA-4, CD40L, and IFN-γ which induce IDO expression suggesting T cell or Treg and IDO expressing APC interactions may act as a negative feedback loop for immune response[5]. These studies indicate that accommodation does not occur through HO-1 alone. It may work through more than one mechanism involving IDO or through an entirely different unknown pathway.

We were unable to determine the mechanism of accommodation in DBA to B6 murine renal transplants. The pathway proved to be too complex to resolve through one molecule (HMO-1), but this does not mean that HMO-1 does not play an important role in the system or that it is not up-regulated during accommodation. Perhaps with further research looking at isolated cells and closer genetic analysis of T cells this process may be revealed. Such information may prove to be valuable in understanding accommodation in transplant patients and developing treatments for them.

References:

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Figure legend:

Figure 1. **Expression of HMOX-1 and foxp3 samples in DBA to B6 murine renal iso- and allo-grafts**. Iso and allo renal grafts were harvested at four time points (d0, 30, 60, 150) and RNA was isolated and reverse transcribed. The cDNA was then quantitave real- time PCRed using primers for (A) FoxP3 and (B) HMOX-1 and run on a 2.0% agarose gel. GAPD was also polymerized as a normalization control.

Figure 2. **Quantification of relative HMOX-1 levels in murine renal iso- and allografts**. Quantitative real-time PCR was run on cDNA samples obtained during the time points indicated using specific primers for HMOX-1 and GAPD. GAPD acted as a positive control for DNA integrity and was used to normalize the relative quantity of HMOX-1 in each sample was normalized to the relative quantity of GAPD.

Figure 3. **Quantification of relative HMOX-1 levels in murine renal iso- and allografts**. cDNA samples taken from the four time points indicated was polymerized using the quantitative real-time PCR method using primers for FoxP3 and GAPD. The relative quantity of FoxP3 in each sample was normalized to the relative quantity of GAPD

Figure 1a:

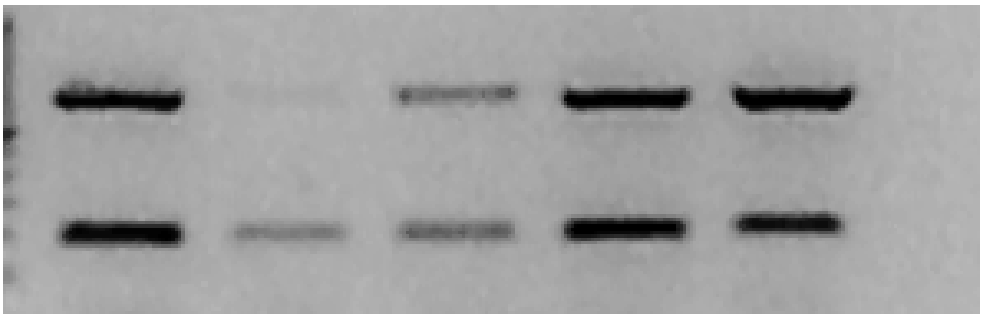

Figure 1b:

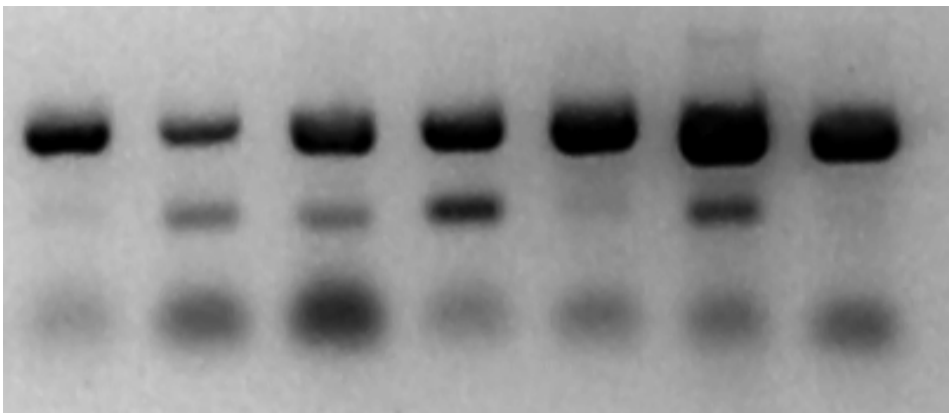

Figure 2:

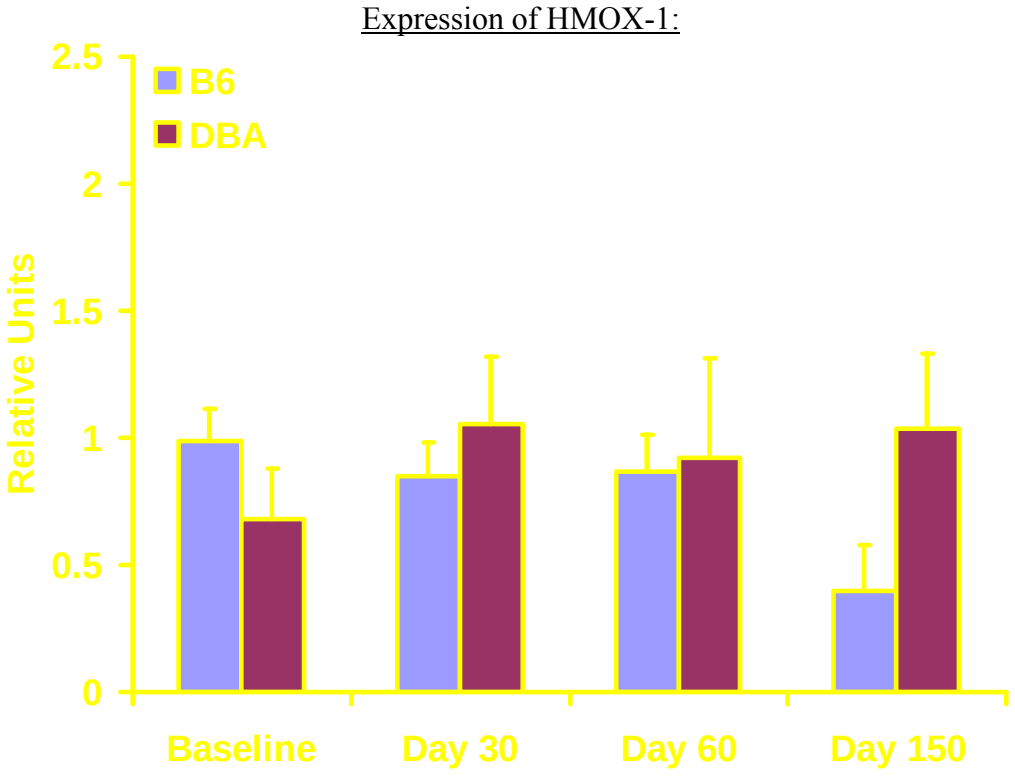
Expression of HMOX-1:
2.5
2
1.5
1
0.5
0
Relative Units
B6
DBA
Baseline
Day 30
Day 60
Day 150

Figure 3:

Expression of FoxP3: